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Immunogold Technique Applied to Simultaneous Identification of T6 and HLA-DR Antigens on Langerhans Cells by Electron Microscopy

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A double-labeling immunogold technique in electron microscopy and specific monoclonal antibodies to surface antigens of Langerhans cells (OKT6 and BL_2) were applied to assess directly the coexpression of two cell surface antigens (T6 and HLA-DR antigens) in a heterogeneous epidermal cell suspension. Electron microscopic examination of double-labeled cells revealed that all Birbeck granule-containing Langerhans cells bound OKT6 and BL_2. The preparation of markers with colloidal gold particles and the procedure for double labeling are described. Several problems related to the steric hindrance and current artifacts are illustrated by micrographs and also discussed.

In the recent past it has been clearly shown that human epidermal cells (EC) consist of keratinocytes, melanocytes, and Langerhans cells (LC). Langerhans cells express various membrane and cytoplasmic markers that allow their distinction from other EC. They express the HLA-DR surface antigens [1–4] which may be identified by the use of anti-HLA-DR monoclonal antibodies (MCA) [5]. Fithian et al [6] and Murphy et al [7,8] have demonstrated that OKT6 MCA, which reacts with a single antigen on the surface of human cortical thymocytes, specifically binds to LC in skin sections [9,10] and in EC suspensions [11,12]. Moreover, the reactivity of normal human LC with MCA specific for T4 antigens usually shared by circulating helper T lymphocytes [13] has been reported [14, 15]. These immunologic markers are expressed by other cells of the monocyte-histiocyte series and by some lymphocytes [16]. Therefore, the identification of LC rests on the electron microscopic demonstration of a cytoplasmic structure, the Birbeck granules [17].

Although LC, which compose 2–5% of the EC population, may be consistently identified with OKT6 MCA in skin sections, only a subset of these are identified by anti-HLA-DR MCA [10,18,19]. Methods using anti-HLA-DR MCA to identify LC in normal EC suspensions have yielded numbers approximately equal to the expected number of OKT6-positive LC [20,21]. By using a combination of existing MCA that react with distinct antigens and a number of complementary techniques, discrete subsets of dendritic cells have been identified within heterogeneous EC populations [21]. These microscopic analyses included simultaneous labeling of reactive cells in sections, in frozen biopsies, and in cell suspensions using various combinations of MCA or antisera conjugated with fluorochromes (fluorescein, tetramethylrhodamine) [6,21,22] or with enzymes (peroxidase) [23]. Immunoelectron microscopic identification of T6 or HLA-DR antigens on LC has been performed using the immunoperoxidase [7–11,12] or immunogold technique [24–26]. One limitation of these electron microscopic methods is the use of a single MCA per EC suspension or skin section.

Proteins such as immunoglobulins can strongly be adsorbed on negatively charged gold particles while maintaining binding activity toward their epitopes. It was therefore demonstrated that T6 and HLA-DR antigens could be localized with gold markers labeled with a suitable antibody by either a direct or an indirect immunogold technique [15,24–26]. Since the first report by Faulk and Taylor [27], this immunogold method has been extensively used [28]. Various advantages of the method were noted: the absence of nonspecific adsorption of gold granules on the cell surfaces, the possibility of poststained ultrathin sections without changes in immunolabeling contrast, and the ability of a quantitative approach of surface antigens based on the direct count of particles located in close apposition to the plasma membrane [15,26,28,29]. Furthermore, colloidal gold allows multiple labeling using gold particles of different sizes [30,31].

In this study, double immunogold labeling electron microscopy was applied to the analysis of LC-enriched EC suspensions. By using this approach it was possible to assess directly the coexpression of 2 cell surface antigens (T6 and HLA-DR antigens) in heterogeneous EC populations. We have demonstrated that OKT6 MCA and anti-HLA-DR MCA react with cells in the epidermis exhibiting the typical ultrastructure of LC. Some experimental points of immunogold labeling procedure are discussed.

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Abbreviations:

Au_5: gold particles 5 nm in diameter
BL_2-Au_5: 5-nm colloid labeled with BL_2 monoclonal antibody
BSA: bovine serum albumin
EC: epidermal cell(s)
FCS: fetal calf serum
LC: Langerhans cell(s)
MCA: monoclonal antibody
PBS: phosphate-buffered saline
TBS: 0.15 M NaCl-0.02 M Tris, pH 7.4, containing 0.5 mg/ml Carbowax 20-M and 0.02% sodium azide
TBS-BSA: TBS containing 1 mg/ml bovine serum albumin
TC199-FCS: TX199 medium supplemented with 20% fetal calf serum

MATERIALS AND METHODS

Monoclonal Antibodies

Two MCA were used: (1) OKT6 is a MCA produced by Ortho Pharmaceutical Co, Raritan, New Jersey, using hybridoma technology. OKT6 has previously been shown to react with 70% of human cortical thymocytes but not with peripheral T cells. Anti-T6 antibody also reacts with intraepidermal LC [7]. (2) Bl_2 is a MCA produced by J. Brochier, the specificity of which was shown to be identical to that of monomorphic anti-HLA-DR. It belongs to the IgG_{2b} isotype and was produced by mouse hybridoma [5,12]. Control ascitic fluid was obtained from animals injected with nonsecreting hybridoma cells.

Epidermal Cell Suspensions

Suspensions of EC were prepared from freshly removed normal human skin (reduction mammoplasty). Trimmed skin was split-cut with a keratotome set at 0.1 mm. The resulting slices were treated for 1 h at 37°C with 0.3% trypsin (Difco, Detroit, Michigan) in buffer

(0.68% NaCl, 0.04% HCl, 0.1% glucose, 0.22% $NaHCO_3$, pH 7.3). The epidermis was separated from the dermis with fine forceps. The isolated epidermal samples were gently teased apart in minimum essential medium (MEM, Gibco, Glasgow) supplemented with 20% fetal calf serum (FCS). The suspension was filtered through a double layer of gauze. The suspended cells were centrifuged for 20 min at 400 *g*. The collected cells were resuspended in TC199 medium (Gibco) supplemented with 20% FCS (TC199-FCS). Following a triple wash in the TC199-FCS, the cells were counted and the viability assessed by trypan blue exclusion. Viability, after trypsinization was 85% or higher.

LC enrichment was obtained by Ficoll-Hypaque (Pharmacia France, Le Chesnay, France) sedimentation. Dispersed skin cells (4×10^6 cells/ml) in TC199-FCS were layered on the gradient and centrifuged for 30 min at 400 *g* at 4°C. The lymphoid-like cells at the interface were washed and resuspended in phosphate-buffered saline (PBS), pH 7.2. Viability was 95% or higher.

Preparation of Gold Marker (BL_2-Au_5)

Gold granules of 5 nm (Au_5) are produced with white phosphorus according to the technique of Horisberger [28]. As small variations of size may occur, colloidal gold must be calibrated by measuring at least 100 particles from electron micrographs and comparing them to standard particles. The gold particles with an average diameter of 5.2 nm (SD = 0.9 nm) were adsorbed with the immunoglobulin fraction of BL_2 MCA according to the procedure of Horisberger [28].

Gold marker BL_2-Au_5 was dispersed in 0.15 M NaCl-0.02 M Tris, pH 7.4, containing 0.5 mg/ml of both sodium azide and Carbowax 20-M. One control consisting of unreactive mouse immunoglobulin fraction (essentially IgG_{2b} isotype) was coupled to Au_5 gold particles to demonstrate the specificity of the direct labeling.

Double Immunogold Labeling of LC-Enriched EC Suspensions

LC-enriched EC suspensions were fixed by 3% paraformaldehyde in PBS. Cell suspensions were suspended and washed in 0.15 M NaCl-0.02 M Tris, pH 7.4, containing 0.5 mg/ml Carbowax 20-M and 0.02% sodium azide (TBS). EC suspensions (5×10^6 cells/ml) were successively labeled with OKT6 MCA plus G40-labeled goat antimouse immunoglobulins (2-step method to reveal T6 antigen) and with Au_5-labeled BL_2 MCA (1-step method to reveal HLA-DR antigens). The labeling procedure was as follows:

(1) TBS containing 10 mg/ml bovine serum albumin (BSA) for 1 h at room temperature; (2) TBS containing 1 mg/ml BSA (TBS-BSA), 2 washes; (3) OKT6 MCA diluted 1:5 in TBS-BSA for 45 min at 37°C; (4) TBS-BSA, 2 washes; (5) goat antimouse immunoglobulin labeled with gold granules of 40 nm size (GAM 40, Janssen Pharmaceutica NV, Beerse, Belgium) diluted 1:2 in TBS-BSA for 45 min at 37°C; (6) TBS-BSA, 2 washes; (7) normal mouse serum diluted 1:20 in TBS-BSA for 45 min at 37°C; (8) TBS-BSA, 2 washes; (9) BL_2-Au_5, A_{520nm} = 3.2 diluted in TBS-BSA for 45 min at 37°C. The cells were finally washed in TBS-BSA, in TBS, and subsequently postfixed for 20 min with 2% glutaraldehyde in cacodylate buffer, then for 20 min with 1% osmium tetroxide, and embedded in Epoxy medium. Ultrathin sections were examined after poststaining with lead citrate and uranyl acetate with a Philips EM 300 electron microscope (kindly provided by Centre de Microscopie Electronique, CMEABG, University Lyon I, Villeurbanne).

To demonstrate the specificity of the labeling, controls consisted of (1) use of mouse IgG_1 immunoglobulins instead of OKT6 MCA, (2) use of uncoupled gold particles stabilized by Carbowax 20-M instead of GAM40, (3) use of unreactive IgG_1-Au_5 instead of OKT6 MCA plus GAM40, and (4) use of unreactive IgG_{2b}-Au_5 instead of BL_2-Au_5. All the solutions were filtered through a Millipore filter (0.45-μm size).

RESULTS

Double Labeling of LC with OKT6 MCA and BL_2 MCA in Cell Suspensions

Three EC suspensions enriched for LC by Ficoll sedimentation were sequentially labeled with OKT6 MCA plus G40-labeled goat antimouse immunoglobulins to indicate the presence of T6 antigen on cell membrane and with Au_5-labeled BL_2 MCA to indicate the presence of HLA-DR antigens (Fig 1).

All the examined labeled suspensions were identified by the presence of variable numbers of probes of electron-dense gold particles of 40 nm and 5 nm scattered along the cell surface. Whatever the size of gold particles, the labeling appeared as

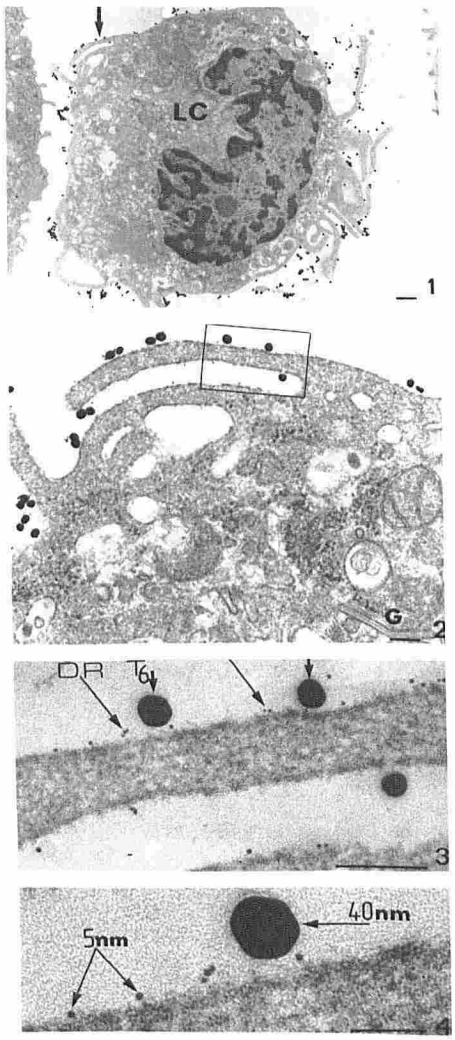

FIG 1. *1*, Double immunogold labeling of human Langerhans cells (*LC*). Isolated dendritic epidermal cells were labeled successively with 40-nm gold granules indicating the presence of T6 antigen and with 5-nm gold granules labeled with anti-HLA-DR MCA (BL_2). Note the absence of gold particles on an adjacent keratinocyte. *Arrow* indicates area considered in *2*, *3*, and *4*. *2*, The OKT6 and BL_2 reactive EC showing ultrastructural characteristics of LC. *G* = Birbeck granules unstained. *Box* indicates area considered in *3* and *4*. *3 and 4*, The 40-nm and 5-nm gold particles do not interact with each other and are exclusively located in close apposition with the cytoplasmic membrane. *Bars*: *1* = 0.5 μm; *2 and 3* = 0.1 μm; *4* = 0.05 μm.

single particles or small clusters of granules in direct apposition with the plasma membrane. When serial electron micrographs were examined, both probes (40 nm and 5 nm particles) were found in different planes, thus indicating they had not interacted and are bound by antigens spatially separated (Fig 1). No gold granules were observed within the cytoplasm of the labeled cells. All the cells containing cytoplasmic Birbeck granules, characteristic of LC, expressed T6 and HLA-DR antigens revealed by the presence of 40 nm and 5 nm gold granules. Although certain labeled cells showed a strong reactivity with anti-HLA-DR BL_2 MCA, Birbeck granules localized near the cell membrane were always negative for the presence of small gold particles. Keratinocytes and melanocytes were negative with the two MCA used in this study.

The incubation with normal mouse serum following the indirect reaction did not decrease or abolish the reactivity of BL_2-Au_5 with cell membrane HLA-DR antigens but prevented interference between 40-nm and 5-nm gold granules. In control experiments in which unreactive mouse IgG_1 was substituted

for OKT6 MCA, the number of small gold particles (5 nm) bound to the cell surface was similar to that of a double-labeled T6-positive, HLA-DR-positive cell. Unreactive IgG_1-Au_5 and IgG_{2b}-Au_5 did not bind to fixed EC.

Additional Controls

Such a micrograph of double labeling (Fig 1) requires precise experimental procedures to establish the specificity of the system and avoid false interpretation.

In our previous experiments, we investigated various undesirable artifacts such as aggregates or coagulation of gold probes either bound to the cell membrane or dispersed in the cell suspension (Fig 2-2). The specificity of a labeling of EC in suspension without cell-membrane permeabilization must be only limited by the accessibility of surface antigens. If the EC morphology (keratinocytes or LC) has been altered, unspecific reactivity was noted between MCA and intracytoplasmic structures especially with small gold particles (Fig 2-*1*). Moreover, cell aggregation and/or adherence of one cell to another restricted contact with the reagents.

DISCUSSION

In this study, we performed a double immunogold labeling with 5- and 40-nm colloidal gold particles for a better identification of LC surface antigens. This immunogold labeling confirms that dendritic EC express a distinctive surface differentiation antigen identified by OKT6 MCA which coexists with, but is different from, HLA-DR antigens on their membrane. Every Birbeck granule-containing cell was double labeled.

The present result confirms the coexistence of T6 antigen and HLA-DR antigens on LC surface but does not allow the comparison of densities of membrane determinants recognized by OKT6 and BL_2 MCA. In such a double labeling no direct relationship can be established between the number of particles of two different sizes bound through a 1-step and a 2-step method and the number of antibody binding sites.

If a double direct reaction may be considered as a time-saving procedure and lead to less nonspecific labeling, it does not allow a double estimation of the distribution of membrane antigenic sites by quantification of bound gold granules. The 1-step method seems to be more restricted by steric hindrance than the 2-step method which usually achieves a more intense labeling [28]. Furthermore, the intensity of labeling is limited by the size of the probe. In our experiment we first performed an indirect immunogold labeling with OKT6 MCA plus G40 goat antimouse immunoglobulins. This method had the advantage that, upon binding, the OKT6 antibody could protrude from its binding site from cell membrane providing greater stereochemical accessibility to the larger gold complex [32] and favored the accessibility of the membrane HLA-DR sites to the smaller BL_2-Au_5 complex.

A limitation of a labeling performed on cell suspensions without cell membrane permeabilization was that only surface antigens could be studied. To demonstrate HLA-DR antigen synthesis by LC, ultrathin sections of fixed cells or tissues would be more suitable for localizing intracellular antigens by means of immunogold cytochemistry. By preserving cell morphology and reducing nonspecific adsorption with use of BSA in the buffer, gold markers may bind nonspecifically to only a very low extent.

Several artifacts may produce false interpretation such as coagulation of colloidal gold and aggregates of proteins or cells. The presence of aggregates may be attributed to (a) the formation of particle aggregates of the gold marker occurring during its preparation by addition of large-size aggregates in the protein solution; (b) the release of antigens from insufficiently fixed cell surface; and (c) the presence of aggregates in the MCA solution applied in the first step of the indirect immunogold method. Furthermore, small clusters of 3 or 4 particles may occur but not be uncommon with antibody molecules although the vast majority of the marker occurred as single particles.

This study demonstrates the importance of varying the size of granules used in immunogold labeling for a quantitative analysis of the expression of HLA-DR antigens and the coexpression of T6 and HLA-DR antigens on LC. Studies are in progress to determine the proportion of LC expressing different densities of antigens in order to establish the exact relationship between LC, the so-called indeterminate cells of the epidermis, and the other epidermal cells expressing different densities of HLA-DR antigens. The fact that cell subpopulations can be distinguished in terms of a heterogeneous membrane antigen distribution could suggest a difference in the capacity of function. Further work will be needed to enrich each LC subpopulation and elucidate their respective immunoelectron microscopic characteristics and function in mixed skin cell-lymphocyte reaction.

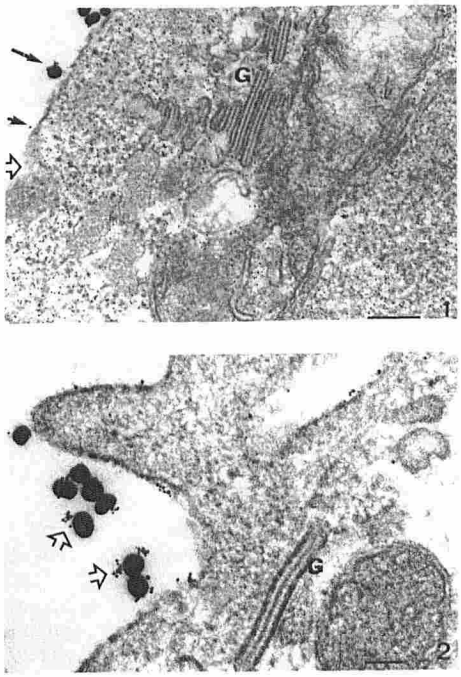

FIG 2. Artifacts occurring during the double immunogold labeling with OKT6 and BL_2 MCA of Langerhans cells. *1*, Unspecific reaction between BL_2-Au_5 and intracytoplasmic structures due to the alteration of the plasma membrane (*white arrow*). *Black arrows* indicate gold particles fixed in the cell membrane. *G* = Birbeck granule. 2, Example of interference (*arrows*) between 40-nm and 5-nm colloidal gold granules prevented by adding normal mouse serum after the complex OKT6/G40 goat antimouse. *G* = Birbeck granule. Bars: *1* = 0.1 μm; *2* = 0.05 μm.

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